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FORM PTO-1390 (REV. 12-2001)

U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: 45419

U S APPLICATION NO (If known, see 37 CFR 1 5: 10/088079

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA00/01096	September 21, 2000	September 22, 1999

TITLE OF INVENTION TRANSGENIC MANIPULATION OF SN-GLYCEROL-3-PHOSPHATE AND GLYCEROL PRODUCTION WITH A FEEDBACK DEFECTIVE GLYCEROL-3-PHOSPHATE DEHYDROGENASE GENE

APPLICANT(S) FOR DO/EO/US

ZOU, Jitao et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ■ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ■ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   - b. ■ has been communicated by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☐ is attached hereto.
   - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ have been communicated by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ■ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (unsigned)
10. ☐ An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ■ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ■ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ■ Other items or information:
    1) International Preliminary Examination Report dated December 14, 2001
    2) Statement Under 37 CFR 1.821(f)
    3) Credit Card Authorization Form
    4) Power of Attorney (unsigned)

JC13 Rec'd PCT/PTO 21 MAR 2

U.S. APPLICATION NO (if known, see 37 CFR 1.5) 10/088079	INTERNATIONAL APPLICATION NO PCT/CA00/01096	ATTORNEY'S DOCKET NUMBER 45419

21. ■ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

	CALCULATIONS	PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ■ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	39 - 20 =	19	x **$18.00**	$ 342.00	
Independent claims	5 - 3 =	2	x **$84.00**	$ 168.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$	

TOTAL OF ABOVE CALCULATIONS =	$ 1,530.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +	$	
SUBTOTAL =	$	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).	$	
TOTAL NATIONAL FEE =	$ 1,530.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$	
TOTAL FEES ENCLOSED =	$ 1,530.00	
	Amount to be refunded:	$
	charged:	$

a. ☐ A check in the amount of $ ______________ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. ______________ in the amount of $ __________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☐ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. __________ . A duplicate copy of this sheet is enclosed.

d. ■ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO.

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33,319
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10/088079
JC13 Rec'd PCT/PTC 21 MAR 2002

IN THE UNITED STATES
PATENT AND TRADEMARK OFFICE

Applicant : ZOU, Jitao et al.

Serial No. :

Filed :

Title : TRANSGENIC MANIPULATION OF SN-GLYCEROL-3-PHOSPHATE AND GLYCEROL PRODUCTION WITH A FEEDBACK DEFECTIVE GLYCEROL-3-PHOSPHATE DEHYDROGENASE GENE

Art Unit :

KIRBY EADES GALE BAKER
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The Hon. Commissioner of Patents
And Trademarks,
Washington, DC 20231 U.S.A.

Dear Sir:

PRELIMINARY AMENDMENT

As a Preliminary Amendment, please amend this application as follows.

IN THE DISCLOSURE

Between lines 1 and 2 of page 1 insert the following wording.

--CROSS-REFERENCE TO RELATED APPLICATION

This application claims the priority right of provisional application Serial No. 60/155,133 filed September 22, 1999 by applicants herein.--

REMARKS

The reason for this amendment is to include a cross-reference to a related application.

Respectfully submitted,

Kimberley A. Lachaine

Kimberley A. Lachaine
Reg. No. 33,319
Our File No. 45419
March 20, 2002

Transgenic manipulation of sn-glycerol-3-phosphate and glycerol production with a feedback defective glycerol-3-phosphate dehydrogenase gene

Field of the invention

The invention relates to the field of plant genetic engineering. More specifically, the invention relates to methods for manipulating the glycerol-3-phosphate metabolism of a plant by expressing in the plant a gene for a feedback defective glycerol-3-phosphate dehydrogenase.

Background of the invention

Glycerol-3-phosphate dehydrogenase (GPDH) (EC 1.1.1.8) is an essential enzyme for both prokaryotic and eukaryotic organisms. It catalyses the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G-3-P) using NADH as reducing equivalent. Plant cells possess at least two isoforms of GPDH, one located in the plastids and the other in the cytosol[1]. The purification of the cytosolic GPDH from spinach has been reported[2]. The product of the reaction catalysed by GPDH, G-3-P, is a precursor for the synthesis of all glycerol lipid species, including membrane and storage lipids. The biosynthetic role of this enzyme in bacteria was established *in vivo* by the isolation of glycerol and G-3-P auxotrophs of *E. coli* mutant strains deficient in its activity[3]. These mutants could not synthesise phospholipid in the absence of supplemental G-3-P.

There are no reports of plant mutants defective in GPDH activity.

In addition to being essential for lipid biosynthesis, GPDH is involved in several other important biological processes. Most notably, GPDH, through consuming NADH and regenerating NAD^+, plays an important role in maintaining cellular redox status. The NAD^+/NADH couple plays a vital role

as a reservoir and carrier of reducing equivalents in cellular redox reactions. For catabolic reactions to proceed, the ratio $NAD^+/NADH$ should be high. Under normal aerobic conditions, excessive NADH is channelled into mitochondria and consumed through respiration. Under anaerobic conditions, GPDH reactions serves as a redox valve to dispose of extra reducing power. In this way, the cellular NAD+/NADH ratio can be maintained at a level allowing catabolic processes to proceed. The expression of the GPDH gene is subject to redox control and induced by anoxic conditions in *Saccaromyces cerevisae*. Deletion of the GPD2 gene (one of the two isoforms of GPDH) results in defective growth under anaerobic conditions[4].

GPDH has also been shown to play an important role in adaptation to osmotic stress in *Saccaromyces cerevisae*. GPDH exerts its role in osmotic and salinity stress response through its function in glycerol synthesis. Glycerol is a known osmo-protectant. It is produced from G-3-P through dephosphorylation by a specific glycerol 3-phosphatase. To respond to a high external osmotic environment, yeast cells accumulate glycerol to compensate for differences between extracellular and intracellular water potentials[5]. The expression of the GPDH gene, GPD1, has been demonstrated to be osmoresponsive[6]. A strain of *Saccaromyces cerevisae* in which the GPD1 gene has been deleted is hypersensitive to NaCl[7]. Accumulation of glycerol as an osmoregulatory solute has been reported in some halophilic green algae including *Dunaliella, Zooxanthellae, Asteromonas* and *Chlamydonas reinhardtii*[8].

The sequence of a cDNA encoding GPDH activity has been reported for the plant *Cuphea lanceolata*[9]. The encoded protein was tentatively assigned as a cytosolic isoform.

To date, there has been no report on the genetic manipulation of plant GPDH.

Summary of the invention

It is an object of the invention to provide a method for expressing in a plant a heterologous glycerol-3-phosphate dehydrogenase.

It is an object of the invention to provide a plant expressing a heterologous glycerol-3-phosphate dehydrogenase, wherein the heterologous glycerol-3-phosphate dehydrogenase is subject to less feedback inhibition than wild type glycerol-3-phosphate dehydrogenase.

It is a further object of the invention to provide a genetically altered plant exhibiting altered fatty acid content in its glycerolipids.

It is a further object of the invention to provide a genetically altered plant exhibiting enhanced tolerance to osmotic stress in comparison to the wild type plant.

It is a further object of the invention to provide a genetically altered plant exhibiting increased stress tolerance in comparison to the wild type plant.

In a first aspect, the invention provides a method for expressing in a plant a heterologous glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase, the method comprising the steps of:

providing a vector comprising a DNA sequence encoding a glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase; and

transforming the plant with the vector.

In a second aspect, the invention provides a plant expressing a heterologous glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase.

In a third aspect, the invention provides a method for producing a genetically altered plant having altered fatty acid content in its glycerolipids, the method comprising the steps of:

providing a vector comprising a DNA sequence encoding a glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase; and

transforming the plant with the vector.

In a fourth aspect, the invention provides a method for producing a plant having increased glycerol and/or glycerol-3-phosphate levels, the method comprising the steps of:

providing a vector comprising a DNA sequence encoding a glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase; and

transforming the plant with the vector.

In a fifth aspect, the invention provides a method for producing a genetically altered plant having increased stress tolerance relative to the wild type, the method comprising the steps of:

providing a vector comprising a DNA sequence encoding a glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase; and

transforming the plant with the vector.

In a sixth aspect, the invention provides a method for producing a genetically altered plant having increased osmotic stress tolerance relative to the wild type, the method comprising the steps of:

providing a vector comprising a DNA sequence encoding a glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase; and

transforming the plant with the vector.

In a seventh aspect, the invention provides a method for increasing the cellular glycerol-3-phosphate dehydrogenase activity in a plant, the method comprising the steps of:

providing a vector comprising a DNA sequence encoding a glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase; and

transforming the plant with the vector.

In an eighth aspect, the invention provides a vector for genetically transforming a plant, wherein the vector comprises a DNA encoding a protein having glycerol-3-phosphate dehydrogenase activity, and the plant, after transforming, exhibits enhanced production of glycerol and/or glycerol-3-phosphate.

Detailed description of the invention

Brief description of the drawings

The invention is illustrated with the aid of the drawings, which show:

FIG. 1 shows the nucleotide sequence and the deduced amino acid sequence of the *Escherichia coli* *gpsA2*^{FR} gene. The point mutation is highlighted and denoted by '*';

FIG. 2 shows a diagram of the *gpsA2*^{FR} plant transformation vector, pGPSA-VI, not drawn to scale;

FIG. 3 shows a southern blot analysis with respect to the *gpsA2*^{FR} gene among the selected independent *Arabidopsis thaliana* transgenic lines.

FIG. 4 shows a northern blot analysis of *gpsA2*^{FR} gene expression in the *A. thaliana* transgenic lines.

FIG. 5 shows the leaf fatty acid profiles of the selected *gpsA2*^{FR} transgenic *Arabidopsis thaliana* lines.

FIG.6 shows the germination rate of the seeds produced by the selected *Arabidopsis thaliana* transgenic lines in ½ MS medium with or without 225 mM NaCl.

FIG. 7 shows the germination rate of wild type *A. thaliana* and transgenic line #13 seeds in ½ MS media supplemented with various concentrations of NaCl.

FIG. 8 shows the performance of the soil-grow transgenic plants under various degree of salinity stress as detailed in Experimental Details.

Due to its role in lipid biosynthesis as well as in the stress responses, an increased GPDH activity in plants is desirable. Transgenic approaches to over express either a plant or a non-plant GPDH gene in a plant can, in principle, be expected to increase GPDH activity. However, there are several advantages inherent in inserting a non-plant gene into a plant genome. It is well established that introducing the same plant gene back to its originating species, even under sense-orientation, can result in a decrease of the over all enzyme activity due to co-suppression. Genes of different origin (heterologous), especially those from evolutionarily distantly related species, can be expected to be free of this impediment. More importantly, proteins of identical enzymatic function are often regulated through different schemes in different species. A heterologous enzyme may potentially be free of controlling factors that inhibit the endogenous enzyme.

The heterologous enzyme that is expressed in the plant, in the method of the invention, may be any glycerol-3-phosphate dehydrogenase that exhibits decreased inhibition of glycerol-3-phosphate production in the plant. Such enzymes are called feed-back defective. In a preferred embodiment, the heterologous enzyme is a glycerol-3-phosphate dehydrogenase having a single amino acid mutation. The mutation should not greatly decrease glycerol-3-phosphate dehydrogenase activity, but should decrease inhibition of the enzyme by glycerol-3-phosphate. One allele of the a *E. coli gpsA* gene, *gpsA2*^{FR}, has been reported to encode an altered version of the GPDH protein defective in feedback inhibition[10]. In a preferred embodiment, the method of the invention uses a vector comprising the gene *gpsA2*^{FR}. The inventors identified a point mutation in the *gspA2*^{FR} sequence: replacement of A by C in

the third nucleotide of codon 255 in *gpsA*. The mutation results in substitution of Glu^{255} (GAA) for Asp^{255} (GAC) in the encoded protein. The sequences of the *gpsA2^{FR}* gene and the deduced amino acid sequence of the gene are shown in FIG. 1. The gene sequence is listed in **SEQ ID NO: 1**, and the encoded protein is listed in **SEQ ID NO: 2.**

The vector may be any vector that is suitable for transforming the plant species used. Examples of suitable vectors include pHS737, pHS738, pRD400 [11]; pBin19 [12]; and pCGN3223 [13].

GPDH is common to the biosynthetic pathway of all plants. The method of the invention can therefore be used with any plant. The inventors chose to use the model plant species *Arabidopsis thaliana*. As a result of the ease with which this plant lends itself to work in both classical and molecular genetics, *Arabidopsis* has come to be widely used as a model organism in plant molecular genetics, development, physiology and biochemistry [14,15,16]. This dicotyledonous plant is also closely related to *Brassica* crop genus and it is increasingly apparent that information concerning the genetic control of basic biological processes in *Arabidopsis* will be transferable to other species[17].

Indeed, there are numerous examples wherein studies of the molecular biology and biochemistry of a particular metabolic pathway or developmental process and the possibility of genetically engineering a plant to bring about changes to said metabolic pathway or process, has first been tested in the model plant Arabidopsis, and then shown to yield similar phenotypes in other plants, particularly crop plants.

Expressing a heterologous GPDH in a plant, according to the method of the invention, leads to altered fatty acid content in the triacylglycerols of the plant. It is often desirable to alter the fatty acid content of glycerolipids to achieve certain desired characteristics in oil seeds. For example, for oils destined for human consumption, it may be wished to increase unsaturated fatty acid content. For other uses, it may be desirable to increase the saturated fatty acid content. The inventors have found that plant transformants

over-expressing the *gpsA2*^{FR} gene produce glycerolipids having an increased proportion of 16 carbon fatty acids and a concomitant decrease of 18 carbon fatty acids.

Due to the relationship of GPDH to glycerolipid synthesis, the method of the invention is particularly suited for use with oil seed bearing plants. The term oil seed bearing plant is meant to encompass any plant or crop from which the oil may be isolated in marketable quantity. Some plants or crops having glycerolipids with particularly interesting fatty acid composition are grown for the production of glycerolipids, even though the lipid content is low (e.g. less than 1 wt%). The method of the invention may be used in such plants to modify the fatty acid content of the glycerolipid. Preferred plants or crops are those having a seed lipid content of at least 1 wt%. Some illustrative examples of oil seed crops are as follows (trivial names are given in parentheses):

Borago officinalis (Borage); *Brassica* species, for example mustards, canola, rape, *B. campestris, B. napus, B. rapa*; *Cannabis sativa* (Hemp, widely uses as a vegetable oil in Asia); *Carthamus tinctorius* (Safflower); *Cocos nucifera* (Coconut); *Crambe abyssinica* (Crambe); *Cuphea* species (*Cuphea* produce medium chain fatty acids of industrial interest); *Elaeis guinensis* (African oil palm); *Elaeis oleifera* (American oil palm); *Glycine max* (Soybean); *Gossypium hiristum* (Cotton – American); *Gossypium barbadense* (Cotton – Egyptian); *Gossypium herbaceum* (Cotton – Asiatic); *Helianthus annus* (Sunflower); *Linum usitatissimum* (Linseed or flax); *Oenethera biennis* (Evening primrose); *Olea europea* (Olive); *Oryza sativa* (Rice); *Ricinus communis* (Castor); *Sesamum indicum* (Sesame); *Soja max* (Soybean – note Glycine max is the major species); *Triticum* species (Wheat); and *Zea maize* (Corn).

GPDH consumes NADH, and therefore plays an important role in maintaining a healthy cellular redox balance. Stress conditions often result in perturbation of plant metabolism, and particularly redox status. Stress conditions include such things as dryness, excessive humidity, excessive heat, excessive cold, excessive sunlight, and physical damage to the plant. Such

agents can lead to higher than normal levels of NADH. Excessive NADH can generate high concentrations of reactive oxygen species (ROS) that are hazardous to proteins and nucleic acids, and may even lead to cell death. An increased GPDH activity, as induced by the method of the invention, improves the capacity of plants to maintain cellular redox balance, thereby leading to an enhanced tolerance to stress.

Another type of stress suffered by plants is osmotic stress. This results when the plant is forced to grow in an environment in which the external water supply has an unusually high concentration of solute. The most usual solutes that are encountered include salts (particularly NaCl), however, in polluted areas, other solutes might be encountered. the method of the invention leads to increased levels of glycerol and/or gycerol-3-phosphate in the tissues of the transformed plant. Glycerol acts as an osmo-protectant, allowing the transformed plant to grow in conditions that would normally not support it.

A heterologous gene encoding GPDH activity can be introduced into genome of plants and expressed using conventional genetic engineering techniques. The most developed methodology for inserting genes into plant genomes is *Agrobacterium tumefaciens* mediated transformation. Other techniques known in the art of introducing DNA into plants include electroporation, chemically-mediated DNA uptake, and the use of microprojectiles.

The invention will be described in more detail with reference to the following examples. The examples serve only to illustrate the invention.

Specific embodiments

a. Molecular Biological Techniques

For a general description of some of the techniques used, see Ausebel *et al* *Current protocols in Molecular Biology*, Vols 1, 2, 3, (1995) New York: Wiley, incorporated herein by reference.

b. Identification of the point mutation of the *gpsA2^{FR}* gene from *Escherichia coli* strain BB26R.

In order to investigate the structure of the *gpsA2^{FR}* gene, the inventors synthesised two primers, TTAGTGGCTGCTGCGCTC (GPSA3, **SEQ ID NO: 3**) and AACAATGAACCAACGTAA (GPSA5, **SEQ ID NO: 4**), complementary to the sequences corresponding to the 3′ and 5′ end of the *gpsA* gene, respectively. PCR amplifications were performed with template DNA isolated from wild type *E. coli* K12 and from strain BB26R, respectively. The BB26R strain harbouring the *gpsA2^{FR}* allele can be obtained according to Cronan *et al.*. The PCR products were purified with QIAquick™ PCR purification Kit (Qiagen™) and fully sequenced. The sequences of *gpsA* (wild type) and *gpsA2^{FR}* (mutant) were compared through sequence alignment using the computer program DNAstar™.

c. Construction of a plant transformation vector for *gpsA^{FR}*

Primers GAGAGCTCTTAGTGGCTGCTGCGCTC (GPSA31, **SEQ ID NO: 5**) and GAAGAAGGATCCAACAATGAACCAACGTAA (GPSA51, **SEQ ID NO: 6**) were designed according to the sequence of *gpsA2^{FR}*. At the 5′ end of GPSA31, a *SacI* restriction site was added, while a *BamHI* restriction site was added at the 5′ end of GPSA5. The primers were used to perform PCR amplification of the *gpsA2^{FR}* sequence. The PCR products were purified with QIAquick™ PCR purification Kit (Qiagen) and digested with *SacI/BamHI*. The *SacI/BamHI* digested *gpsA2^{FR}* DNA fragment was subsequently inserted into the *Agrobacterium* binary vector pBI121 (Clontech) to replace the *SacI/BamHI* region covering the GUS gene. The resultant plant transformation vector is designated as pGPSA-VI (deposited August 31, 2000, at the American Type Culture Collection, 10801 University Blvd. Manassa, VA 20110-2209, accession no. PTA-2433). The *gpsA2^{FR}* gene expression cassette in pGPSA-VI contains the *gpsA2^{FR}*-encoding region driven by the constitutive 35S promoter. Its 3′ end is flanked by the NOS terminator. The junction region between the 35S promoter

and the *gpsA2*^{FR} encoding sequence in pGPSA-VI was confirmed through sequencing. The gpsA2^{FR} protein will thus be expressed in all plant tissues including vegetative and reproductive (seed) tissues once the gene expression cassette is incorporated into the plant genome.

d. Plant Growth Conditions

Arabidopsis thaliana was chosen as the plant host to test the effect of the *gpsA2*^{FR} gene since it is widely recognised as a laboratory model plant for genetic and biochemical studies. Moreover, *A. thaliana* in many aspects resembles *Brassica napus*, and is considered an oilseed plant. Genetic manipulations that are successful with *A. thaliana* can be applied to other species[18]. All *A. thaliana* control and transgenic plants were grown at the same time, in controlled growth chambers, under 16 hr fluorescent illumination (150-200 $\mu E.m^{-2}.sec^{-1}$), 8 hr dark at 22 °C., as described previously[19].

e. Plant Transformation

Plasmid pGPSA-VI was introduced into *Agrobacterium tumefaciens* strain GV3101 bearing helper nopaline plasmid pMP90, via electroporation. Wild type *A. thaliana* plants of ecotype Columbia were grown in soil. Plants one week after bolting were vacuum-infiltrated over night with a suspension of *A. tumefaciens* strain GV3101 harbouring pGPSA-VI [20].

After infiltration, plants were grown to set seeds (T1). Dry seeds (T1) were harvested in bulk and screened on selective medium with 50 mg/L kanamycin. After two to three weeks on selective medium, kanamycin resistant seedlings (T1) which appeared as green were transformed to soil to allow growing to maturity. Seeds (T2) from the T1 plants were harvested and germinated on kanamycin plates to test segregation ratios. A typical single gene insertion event would give rise to a kanamycin resistant/sensitive ratio of 3:1. To further confirm the integration of the *gpsA2*^{FR} gene, DNA was isolated from selected transgenic lines to perform Southern blot analysis with probes prepared with

gpsA2^FR DNA. Total RNA was also isolated for Northern analysis to confirm the expression of the *gpsA2^FR* gene.

f. Fatty acid profile analysis

Lipids were isolated from developing leaves as described by Katavic *et al.* [21]and the fatty acid compositions were analysed by Gas Chromatography.

g. Analysis of plant tolerance towards salinity stress

The salt tolerance of *A. thaliana* ecotype Columbia (wild-type) plants and plants over-expressing the *gpsA2^FR* gene was measured using a protocol reported by Apse *et al* [22]. Pots of wild-type plants and each of the four transgenic lines (designated as #7, #13, #54 and #58) over-expressing *gpsA2^FR* gene were divided into five groups (labelled A through E). The plants were planted in 4′ pots with each pot containing 4 plants. The plants were grown for two-weeks with nutrients-only [22 g of 20:20:20 plant nutrient (Plant Products Co. Ltd., Canada) in 80 litres of water] solutions to ensure even growth of all plants. Afterwards, every alternate day over a 16-day watering regime, 25 ml of a diluted nutrient solution was applied. The control (A) group received the nutrient –only solution with no NaCl supplementation. The remaining groups were watered with nutrient solution supplemented with NaCl. The concentrations of NaCl supplementation were increased stepwise by 50 mM every 4 days for each group, to the indicated maximum: (A) 0 mM NaCl, (B) 50 mM NaCl, (C) 100 mM NaCl, (D) 150 mM NaCl, and (E) 200 mM NaCl. The plants were monitored for their phenotype, flowering time etc.

Seed germination assays were performed with surface sterilised *Arabidopsis* seeds of wild type and selected T3 transgenic lines sown in Petri dishes containing 20 ml half strength MS medium[23], supplemented with B5 vitamins and 2% sucrose. For the salt stress germination assay, various concentrations of NaCl were added. Cultures were grown at 22 °C under fluorescent light, 16h light and 8h dark. Seed germination was recorded after a

period of 10 days. The emergence of radicle and cotyledons was considered as evidence of germination.

Results

The *gpsA2^FR* gene has a point mutation that alters one amino acid Residue in the GPDH protein (gpsA2^FR)

The biosynthesis of G-3-P in *Escherichia coli* was initially investigated by Kito and Pizer[24]. The *gpsA* locus located at minute 71 of the *E. coli* genetic map was determined to be the structural gene for the biosynthetic glycerol-3-phosphate dehydrogenase by Cronan and Bell[25]. The nucleotide sequence and the deduced amino acid sequence of the *Escherichia coli gpsA* gene was reported previously[26]. Biochemical studies on phospholipid biosynthesis mutants indicated that the cellular level of G-3-P must be tightly regulated Bell (1974), *J. Bacteriol.*117, 1065-1076]. The *E. coli* mutant, *plsB*, possesses a glycerol-P acyltransferase with an apparent K_m for G-3-P over 10 times higher than normal. Subsequently, revertants of the *plsB* mutant, BB26R, were identified[27]. The glycerol-3-phosphate dehydrogenase activities of these revertants were about 20-fold less sensitive to feedback inhibition by G-3-P. These feedback resistant *gpsA* alleles were named *gpsA2^FR*. The molecular mechanism behind the gpsA2^FR protein was unknown. The *gpsA2^FR* gene was cloned from strain BB26R and its nucleotide sequence was determined. Sequence analysis indicated that *gpsA2^FR* differs from *gpsA* at only one nucleotide base. The point mutation, a replacement of A from C at the third nucleotide of codon 255 in *gpsA* (FIG. 1) was founded in the *gpsA2^FR* gene. This point mutation resulted in a change of Glu^{255} (GAA) from Asp^{255} (GAC) in the glycerol-3-phosphate dehydrogenase enzyme protein.

It has now been shown that the *gpsA2^FR* gene harbours a point mutation in comparison to the wild type *gpsA* gene. The inventors have demonstrated that the point mutation is the reason why the GPDH enzyme is 20 time less sensitive

to G-3-P feedback inhibition than the wild type. As a result, the cellular G-3-P could reach a level higher than a wild type gpsA could generate.

Introduction of the *gpsA2^{FR}* gene into plant genomes does not affect plant development

A large number of *gpsA2^{FR}* transgenic plants were generated. These transgenic plants (T1) were initially screened for kanamycin resistance in kanamycin supplemented ½ MS medium. All T1 transgenic plants under our growing conditions appeared indistinguishable from wild type *A. thaliana* control, and developed at the same pace as that of the wild type plants when transferred into soil. The fertility and the seed yield were also not affected by the transgene. It thus proved that the integration of the *gpsA2^{FR}* gene did not have any adversary effect on plant growth and reproduction. The segregation ratios of the (T2) seeds from the T1 plants with regard to kanamycin resistance were investigated. Transgenic line #7, #13, #54, #58 were selected for further study since segregation analysis indicated that these lines were single-insertion transgenic lines. To further verify the incorporation of *gpsA^{FR}* gene into plant genome, genomic DNA was isolated from T3 plant seedlings of line #7, #13, #54, #58, respectively. Southern analysis of genomic DNA digested with three different restriction enzymes showed that these lines contain a single copy of the *gpsA2^{FR}* gene, and the transgene is inherently stable (FIG. 4). Northern analysis with RNA extracted from these lines confirmed that the *gpsA2^{FR}* gene is expressed at a high level in these transgenic lines. Therefore, the introduction and expression of the *gpsA2^{FR}* gene into higher plants was accomplished.

***A. thaliana gpsA2^{FR}* transformants have altered fatty acid profiles**

Total lipids were extracted from leaf tissues of transgenic plants as well as wild type control, and the fatty acid compositions were analysed using Gas Chromatography. In order to minimise any difference that might exist during plant development, care was taken to ensure all plant leaves collected were at

the same developmental stage. Reproducible results were obtained with leaves collected from several wild type plants, confirming that there were no significant differences with regard to fatty acid profiles among wild type plants. Data from leaves of the *A. thaliana* transgenic plants, however, indicated that the $gpsA2^{FR}$ gene product affects fatty acid composition. As shown in FIG. 5, $gpsA2^{FR}$ transgenic plants consistently had elevated levels of 16 carbon fatty acids, and proportionately decreased level of 18 carbon fatty acid. Specifically, the transgenic plants showed about a 2-5 % increase of 16:0, and about a 1.5-3.5 % increase of 16:3 fatty acids. Concomitantly, the decrease on 18:2 and 18:3 fatty acids is at a 2-5% range (FIG. 5). Differences between the transgenic plants and the controls are also apparent if the ratios of the sum of 16-carbon (16C) fatty acid versus the sum of 18-carbon (18C) fatty acids are compared. For example, under the growing conditions described, transgenic line #58, line #13 and line #54 had 16C/18C ratios of 0.53, 0.6 and 0.68, respectively, while the ratio in control plants was 0.43. This phenotype is most likely a direct result of an increased supply of G-3-P generated by the high GPDH activity in the transgenic plants. It is consistent with previous report by Gardiner *et al*, in which an increased ratio of 16C/18C fatty acids was observed among newly synthesised fatty acids when elevated amounts of G-3-P were fed to isolated plastids[28].

The $gpsA2^{FR}$ gene improved plant stress tolerance

As stated previously, GPDH consumes NADH and regenerates NAD^+. Lowering cellular [NADH] has beneficial effects on mitochondrial respiration and energy charge. GPDH participates in the control of cellular redox status, and possibly reduces the concentration of potentially damaging reactive oxygen species. Plant cells are known to go through an oxidative burst under stress conditions, often leading to cell death.

The present study revealed that the $gpsA2^{FR}$ transgenic plants possessed enhanced salinity tolerance.

The enhanced salinity tolerance could be observed at different developmental stages. Transgenic plant seeds germinated at the same frequency as that of the non-transgenic control plants on ½ MS medium (FIG. 6, upper panel). However, on media with added salt (Fig. 6, lower panel), the wild type germinated at only about 55%, while transgenic lines #54, #58, #7 and #13 germinated at a rate of 90%, 86%, 87% and 95%, respectively. The germination frequencies of line #13 seeds were further evaluated with various NaCl concentrations. As shown in FIG.7, in all concentrations of NaCl examined, line #13 seeds consistently showed higher germination rates than that of the wild type plant seeds. The most dramatic effect was observed with 250 mM NaCl, in which less than 40% of wild type seeds germinated, while 80% of the line #13 seeds germinated. In neither cases could auxotrophic growth be established from the germinated seeds.

Wild type *A. thaliana* could germinate reasonably well (80%)on medium containing 175 mM NaCl. However, seedling growth and development were severely retarded. In contrast, the growth rate of the transgenic plants was substantially higher. After 6 weeks, wild type plants developed chlorosis on leaf tissues and eventually died, while under the same conditions the transgenic plants still maintain relatively healthy green leaves. Plants growing in soil were also investigated with respect to salinity tolerance. The inventors followed the treatment protocol reported by Apse *et al*[29], designed to mimic field stress conditions. As shown in FIG. 8, the transgenic plants displayed advanced growth and developmental profiles in comparison to those of wild type plants. Most of the wild type plants repeatedly treated with 50 mM NaCl appeared severely stressed with darkened leaf colour. The same treatment did not seem to affect the growth and reproduction of the transgenic lines. Wild type plants ceased to grow and eventually died when solutions containing salt at 100 mM were applied, while the majority of the transgenic plants developed to maturity and produced seeds. When a watering regime was carried out to a salt concentration of 150 mM NaCl, the transgenic plants showed apparent stressed

phenotype, but were still able to produce seeds, albeit with short siliques and very little seed yield. Plants from line # 54 exhibited the most improved salinity among the transgenic lines tested. They produced seeds even when watering reached a salt concentration of 200 mM NaCl.

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REFERENCE: **Patent Deposit**

Escherichia coli BB26R with DNA insert: pGPSA VI assigned PTA-2433.

Date of Deposit: August 31, 2000 Paperwork will be forwarded to you in a few days. An invoice will be sent under separate cover. The Mastercard account of Irene Howe will be charged as follows:

Standard storage/informing	$ 1,100.00
Viability Test	200.00
Total amount to PTA-2433	$ 1,300.00

Tanya Nunnally, Patent Specialist
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What is claimed is:

1. A method for expressing in a plant a heterologous glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase, the method comprising the steps of:
providing a vector comprising a DNA sequence encoding a glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase; and transforming the plant with the vector.

2. A method according to claim 1, wherein the glycerol-3-phosphate dehydrogenase has a single amino acid substitution which renders it feedback defective, while not significantly altering its catalytic ability.

3. A method according to claim 1, wherein the glycerol-3-phosphate dehydrogenase is gpsA2^{FR}.

4. A method according to claim 1, wherein the DNA sequence comprises a DNA sequence encoding the amino acid sequence listed in **SEQ ID NO: 2.**

5. A method according to claim 1, wherein the DNA sequence encoding the glycerol-3-phosphate dehydrogenase comprises the sequence listed in **SEQ ID NO: 1.**

6. A method according to claim 1, wherein the glycerol-3-phosphate dehydrogenase has the amino acid sequence listed in **SEQ ID NO: 2.**

7. A method according to claim 1, wherein the plant is an oil seed bearing plant.

8. A method according to claim 1, wherein the plant is of the genus *Brassica*.

9. A method according to claim 1, wherein the plant is *Arabidopsis thaliana*.

10. A plant expressing a heterologous glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase.

11. A plant according to claim 10, wherein the glycerol-3-phosphate dehydrogenase has a single amino acid substitution which renders it feedback defective, while not significantly altering its catalytic ability.

12. A plant according to claim 10, wherein the glycerol-3-phosphate dehydrogenase is gpsA2^{FR}.

13. A plant according to claim 10, wherein the plant harbours a DNA sequence encoding the amino acid sequence listed in **SEQ ID NO: 2.**

14. A plant according to claim 10, wherein the plant harbours a DNA sequence as listed in **SEQ ID NO: 1.**

15. A plant according to claim 10, wherein the glycerol-3-phosphate dehydrogenase has the amino acid sequence listed in **SEQ ID NO: 2.**

16. A plant according to claim 10, wherein the plant is an oil seed bearing plant.

17. A plant according to claim 10, wherein the plant is of the genus *Brassica*.

18. A plant according to claim 10, wherein the plant is *Arabidopsis thaliana*.

19. A method for producing a genetically altered plant having altered fatty acid content in its glycerolipids, the method comprising the steps of: providing a vector comprising a DNA sequence encoding a glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase; and transforming the plant with the vector.

20. A method according to claim 19, wherein the glycerol-3-phosphate dehydrogenase has a single amino acid substitution which renders it feedback defective, while not significantly altering its catalytic ability.

21. A method according to claim 19, wherein the glycerol-3-phosphate dehydrogenase is gpsA2^{FR}.

22. A method according to claim 19, wherein the DNA sequence comprises a DNA sequence encoding the amino acid sequence listed in **SEQ ID NO: 2.**

23. A method according to claim 19, wherein the DNA sequence encoding the glycerol-3-phosphate dehydrogenase comprises the sequence listed in **SEQ ID NO: 1.**

24. A method according to claim 19, wherein the glycerol-3-phosphate dehydrogenase has the amino acid sequence listed in **SEQ ID NO: 2.**

25. A method according to claim 19, wherein the plant is an oil seed bearing plant.

26. A method according to claim 19, wherein the plant is of the genus *Brassica*.

27. A method according to claim 19, wherein the plant is *Arabidopsis thaliana*.

28. A method according to claim 19, wherein the plant glycerolipid has elevated levels of C16 fatty acids.

29. A method for producing a genetically altered plant having increased stress tolerance relative to the wild type, the method comprising the steps of:
providing a vector comprising a DNA sequence encoding a glycerol-3-phosphate dehydrogenase that is less sensitive to feedback inhibition than wild type glycerol-3-phosphate dehydrogenase; and transforming the plant with the vector.

30. A method according to claim 29, wherein the glycerol-3-phosphate dehydrogenase has a single amino acid substitution which renders it feedback defective, while not significantly altering its catalytic ability.

31. A method according to claim 29, wherein the glycerol-3-phosphate dehydrogenase is $gpsA2^{FR}$.

32. A method according to claim 29, wherein the DNA sequence comprises a DNA sequence encoding the amino acid sequence listed in **SEQ ID NO: 2.**

33. A method according to claim 29, wherein the DNA sequence encoding the glycerol-3-phosphate dehydrogenase comprises the sequence listed in **SEQ ID NO: 1.**

34. A method according to claim 29, wherein the glycerol-3-phosphate dehydrogenase has the amino acid sequence listed in **SEQ ID NO: 2.**

35. A method according to claim 29, wherein the plant is an oil seed bearing plant.

36. A method according to claim 29, wherein the plant is of the genus *Brassica*.

37. A method according to claim 29, wherein the plant is *Arabidopsis thaliana*.

38. A method according to claim 29, wherein the stress is osmotic stress.

39. A vector for genetically transforming a plant, wherein the vector comprises a DNA encoding a protein having glycerol-3-phosphate dehydrogenase activity, and the plant, after transforming, exhibits enhanced biosynthesis of glycerol and/or glycerol-3-phosphate.

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(54) Title: TRANSGENIC MANIPULATION OF SN-GLYCEROL-3-PHOSPHATE AND GLYCEROL PRODUCTION WITH A FEEDBACK DEFECTIVE GLYCEROL-3-PHOSPHATE DEHYDROGENASE GENE

(57) Abstract: The invention provides a method for genetically transforming a plant so that it expresses a glycerol-3-phosphate dehydrogenase that is feed-back defective. The feed-back defective enzyme raises levels of glycerol and glycerol-3-phosphate in comparison to the wild type, leading to increased osmotic stress tolerance, and altered fatty acid content in glycerolipids.

Figure 1

```
  1 atgaaccaacgtaatgcttcaatgactgtgatcggtgccggctcg
    M  N  Q  R  N  A  S  M  T  V  I  G  A  G  S   15
 46 tacggcaccgctcttgccatcaccctggcaagaaatggccacgag
    Y  G  T  A  L  A  I  T  L  A  R  N  G  H  E   30
 91 gttgtcctctggggccatgaccctgaacatatcgcaacgcttgaa
    V  V  L  W  G  H  D  P  E  H  I  A  T  L  E   45
136 cgcgaccgctgtaacgccgcgtttctccccgatgtgccttttccc
    R  D  R  C  N  A  A  F  L  P  D  V  P  F  P   60
181 gatacgctccatcttgaaagcgatctcgccactgcgctggcagcc
    D  T  L  H  L  E  S  D  L  A  T  A  L  A  A   75
226 agccgtaatattctcgtcgtcgtacccagccatgtctttggtgaa
    S  R  N  I  L  V  V  V  P  S  H  V  F  G  E   90
271 gtgctgcgccagattaaaccactgatgcgtcctgatgcgcgtctg
    V  L  R  Q  I  K  P  L  M  R  P  D  A  R  L  105
316 gtgtgggcgaccaaagggctggaagcggaaaccggacgtctgtta
    V  W  A  T  K  G  L  E  A  E  T  G  R  L  L  120
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406 gttatctctggcccaacgtttgcgaaagaactggcggcaggttta
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    P  T  A  I  S  L  A  S  T  D  Q  T  F  A  D  165
496 gatctccagcagctgctgcactgcggcaaaagtttccgcgtttac
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586 aacgttattgccattggtgcggggatgtccgacggtatcggtttt
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631 ggtgcgaatgcgcgtacggcgctgatcacccgtgggctggctgaa
    G  A  N  A  R  T  A  L  I  T  R  G  L  A  E  225
676 atgtcgcgtcttggtgcggcgctgggtgccgaccctgccacctttt
    M  S  R  L  G  A  A  L  G  A  D  P  A  T  F  240
721 atgggcatggcggggcttggcgatctggtgcttacctgtaccgaa*
    M  G  M  A  G  L  G  D  L  V  L  T  C  T  E  255
766 aaccagtcgcgtaaccgccgttttggcatgatgctcggtcagggc
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811 atggatgtacaaagcgcgcaggagaagattggtcaggtggtggaa
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946 tgcggaaaaaacgcgcgcgaggcagcattgactttactaggtcgt
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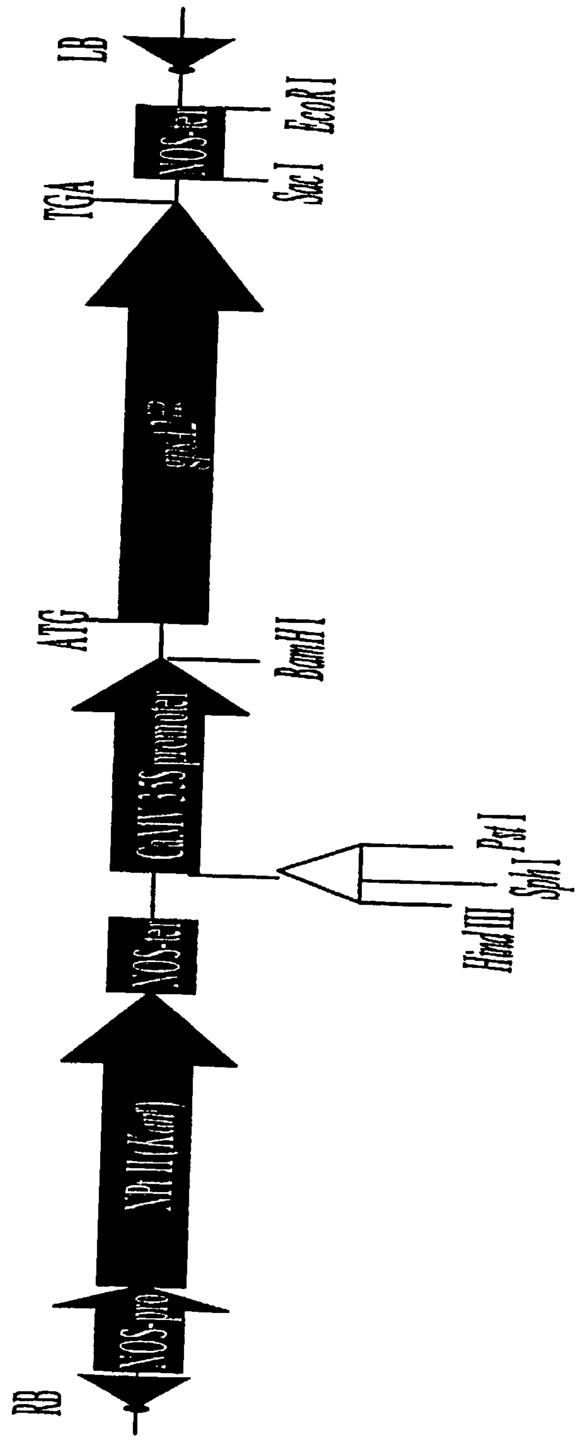
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Sac I
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gus
ATG
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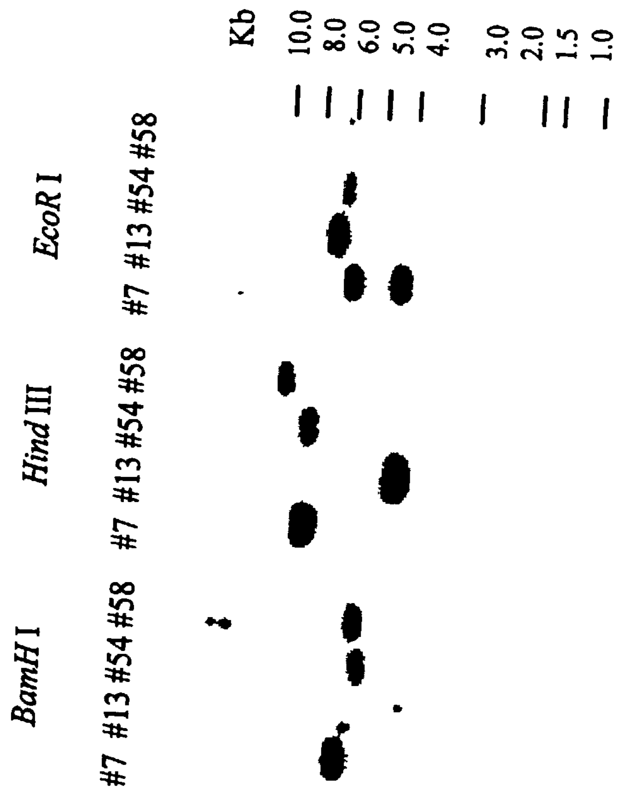
Hind III
NOS-ter
NPT II (Kan^r)
NOS-pro
RB

Figure 2

Figure 3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EcoR I
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5.0
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3.0
2.0
1.5
1.0

Figure 4

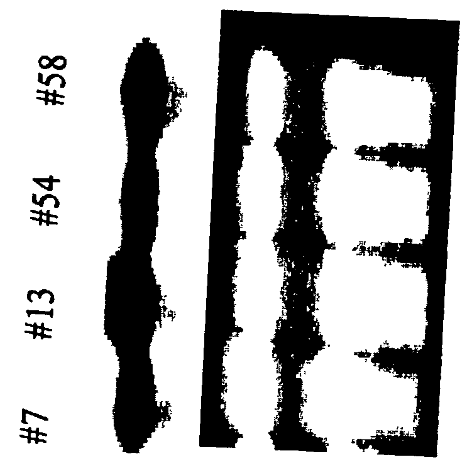
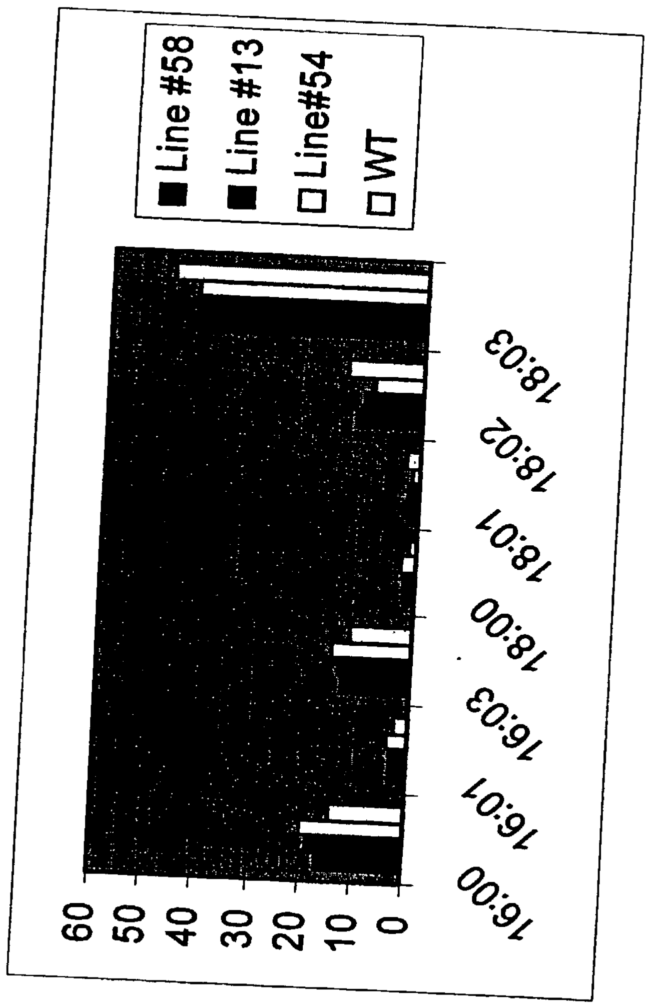


Figure 5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Line #13
Line#54
WT
60
50
40
30
20
10
0
16:00
16:01
16:03
18:00
18:01
18:02
18:03

Figure 6

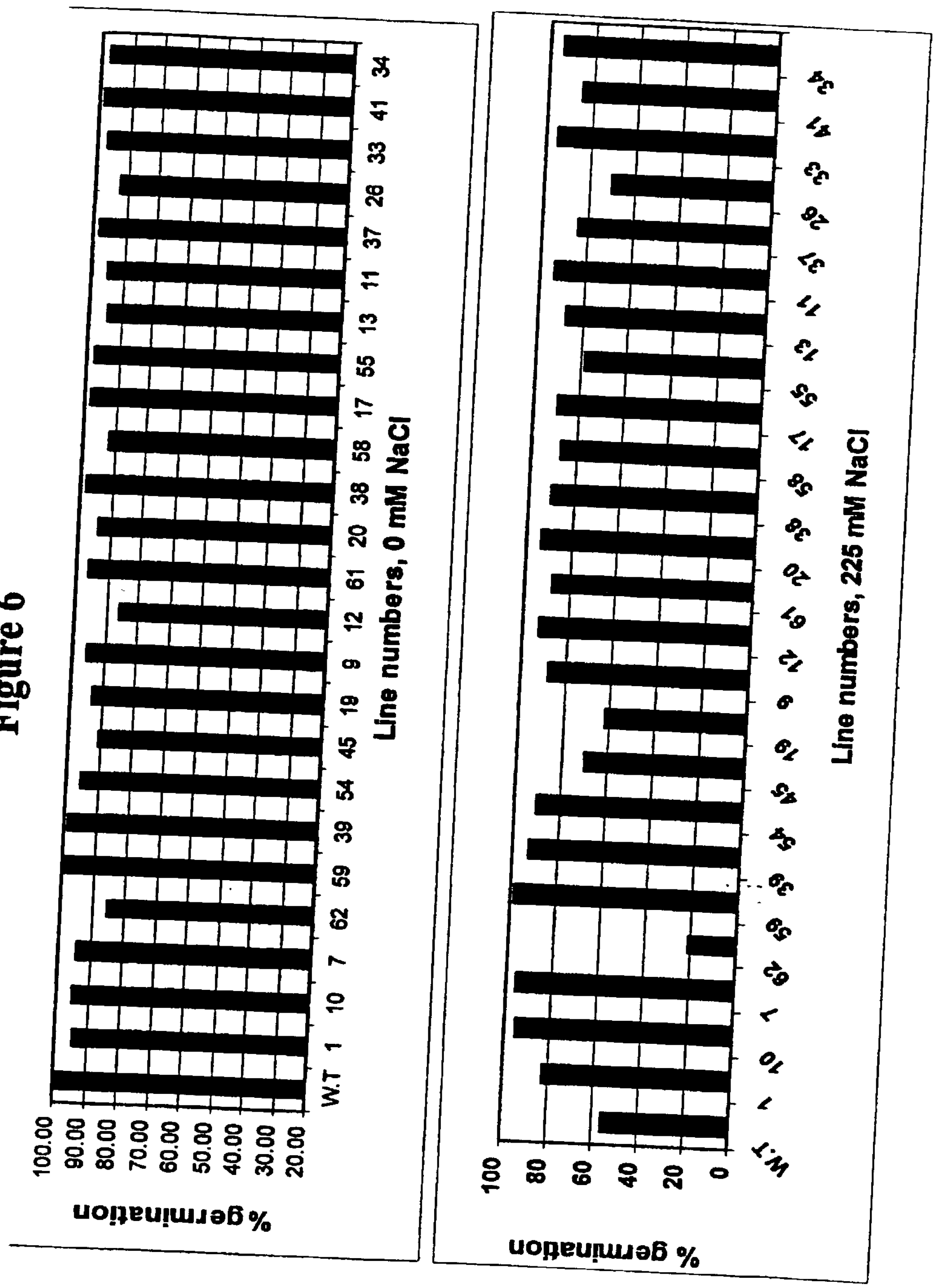

% germination
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
W.T 1 10 7 62 59 39 54 45 19 9 12 61 20 38 58 17 55 13 11 37 28 33 41 34
Line numbers, 0 mM NaCl
% germination
100
80
60
40
20
0
W.T 1 10 7 62 59 39 54 45 19 9 12 61 20 38 58 17 55 13 11 37 26 33 41 34
Line numbers, 225 mM NaCl

Figure 7

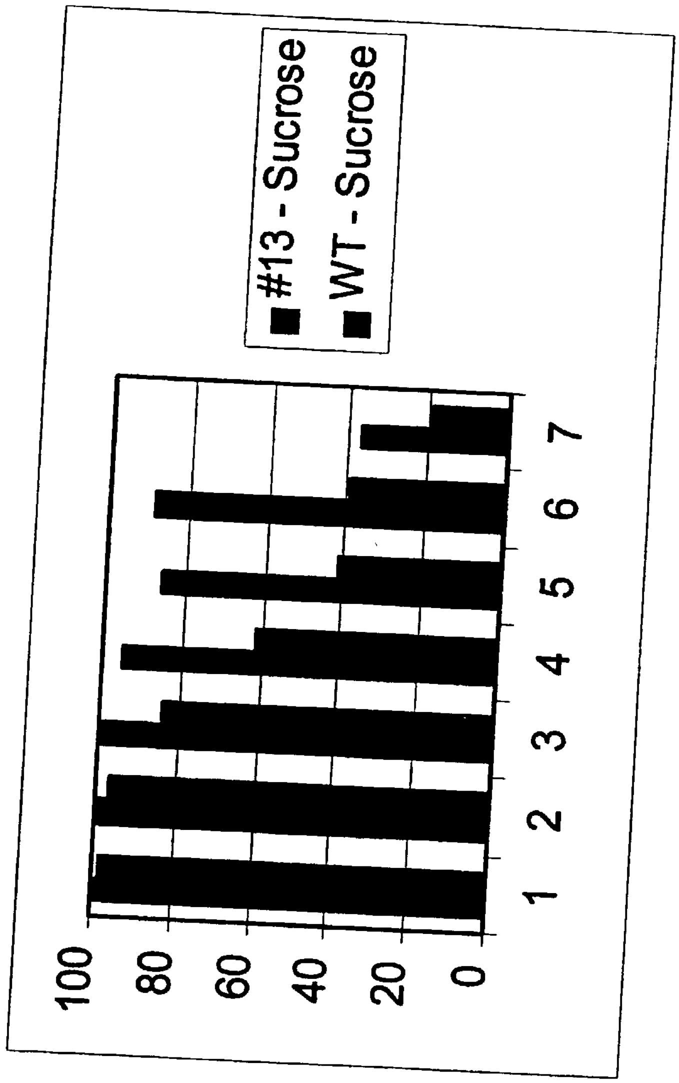
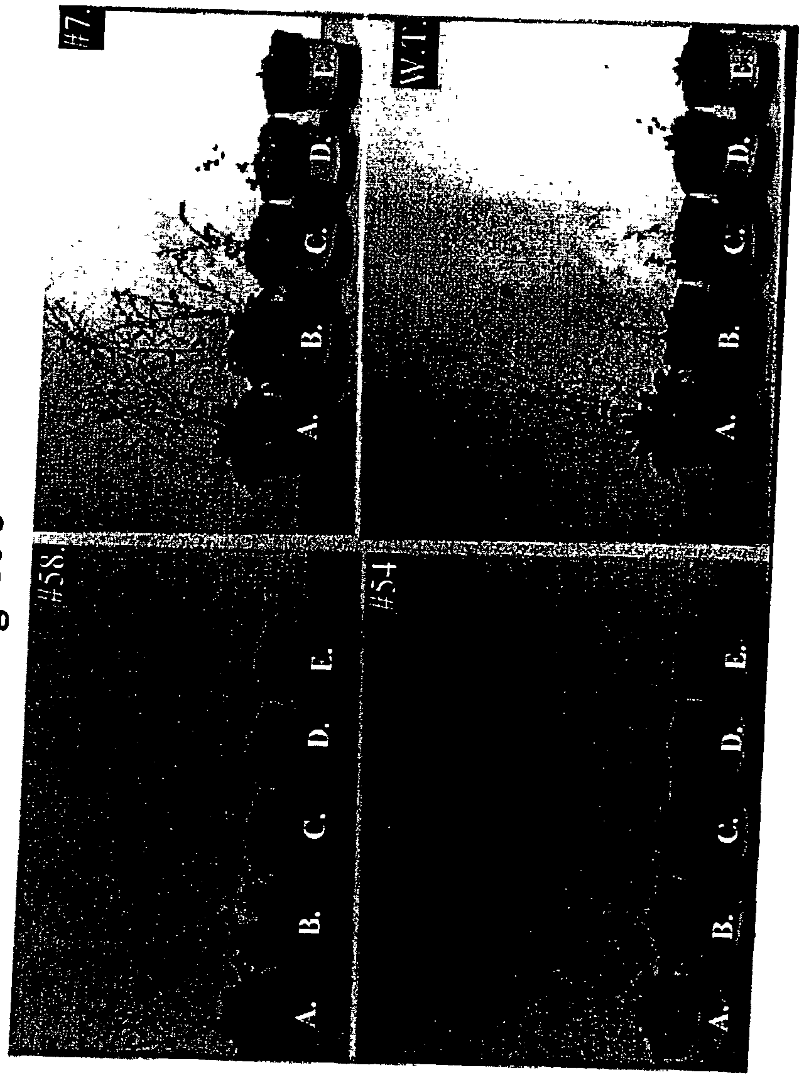


Figure 8



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<110> National Research Council of Canada

<120> Transgenic manipulation of sn-glycerol-3-phosphate and glycerol production with a feedback defective glycerol-3-phosphate dehydrogenase gene

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Cys Asn Ala Ala Phe Leu Pro Asp Val Pro Phe Pro Asp Thr Leu His
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Val Val Val Pro Ser His Val Phe Gly Glu Val Leu Arg Gln Ile Lys
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Pro Leu Met Arg Pro Asp Ala Arg Leu Val Trp Ala Thr Lys Gly Leu
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PTO/SB/01 (10-01)
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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted with Initial Filing **OR** ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	45419
First Named Inventor	ZOU, Jitao et al.
COMPLETE IF KNOWN	
Application Number	10/088,079
Filing Date	March 21, 2002
Art Unit	
Examiner Name	

As the below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original and first inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TRANSGENIC MANIPULATION OF SN-GLYCEROL-3-PHOSPHATE AND GLYCEROL PRODUCTION WITH A FEEDBACK DEFECTIVE GLYCEROL-3-PHOSPHATE DEHYDROGENASE GENE

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☑ was filed on (MM/DD/YYYY) 09/21/2000 as United States Application Number or PCT International Application Number PCT/CA00/01096 and was amended on (MM/DD/YYYY) ________ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	**Country**	**Foreign Filing Date (MM/DD/YYYY)**	**Priority Not Claimed**	**Certified Copy Attached? YES**	**NO**
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

10088075 .071902

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DECLARATION — Utility or Design Patent Application

Direct all correspondence to: ☑	Customer Number or Bar Code Label 02048 PATENT TRADEMARK OFFICE	OR ☐	Correspondence address below
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR : ☐ A petition has been filed for this unsigned inventor

100

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NAME OF SECOND INVENTOR: ☐ A petition has been filed for this unsigned inventor

200

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Mailing Address	1514 MAIN STREET, APT. 8		
City	SASKATOON	State Saskatchewan	ZIP S7J 4C3 / Country CANADA

☑ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.

10088079 .072902

PTO/SB/02A (10-00)
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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 1 of 1

300

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor		
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Inventor's Signature Cyril Periappuram			**Date** April 26 2002
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Mailing Address 2238 MOLECULAR BIOLOGY BUILDING IOWA STATE UNIVERSITY			
City ~~SASKATOON~~ AMES	**State** ~~SK~~ IA	**ZIP** ~~S7K 2M7~~ 50011-3260	**Country** ~~CANADA~~ USA

400

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Inventor's Signature G Selvaraj			**Date** April 24 2001
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500

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor		
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Mailing Address			
City SASKATOON	**State** SK	**ZIP** S7H 5C4	**Country** CANADA

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☐ Page(s)__________ of ______________________________ were not present for scanning. (Document title)

☐ Page(s)__________ of ______________________________ were not present for scanning. (Document title)

— only 8 drawings

☐ ***Scanned copy is best available.***